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(54) Title: METHODS TO IDENTIFY COMPOUNDS THAT MODULATE RAGE



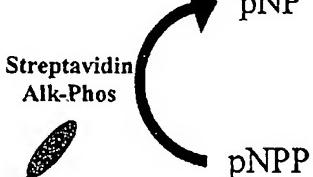
Immobilized Ligand



sRAGE



Primary Antibody

Secondary F(ab')₂
Antibody-biotin**WO 01/92892 A2**

(57) Abstract: Provided are methods to detect modulators of the Receptor for Advanced Glycated Endproducts (RAGE). The invention comprises a method for detection of RAGE modulators comprising: adsorbing a RAGE ligand onto a solid surface; adding a compound of interest and a protein comprising RAGE or fragment thereof, to the preadsorbed ligand; adding an antibody which binds to RAGE or fragment thereof and a secondary antibody which binds to the anti-RAGE antibody; measuring the secondary antibody bound to the anti-RAGE antibody; and comparing the amount of RAGE bound to the ligand in the presence of varying amounts of the compound of interest. In an embodiment, the fragment of RAGE is sRAGE. In one aspect, the invention uses compounds detected by the method for treatment of AGE-related syndromes including complications associated with diabetes, kidney failure, lupus nephritis or inflammatory lupus nephritis, amyloidoses, Alzheimer's disease, cancer, inflammation, and erectile dysfunction.

METHODS TO IDENTIFY COMPOUNDS THAT MODULATE RAGE

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to provisional patent application 60/207,342, filed May 30, 2000.

FIELD OF THE INVENTION

10 The present invention relates to regulation of the Receptor for Advanced Glycated Endproducts (RAGE). More particularly, the present invention describes methods for the rapid, high-throughput identification of modulators of RAGE.

BACKGROUND OF THE INVENTION

15 Incubation of proteins or lipids with aldose sugars results in nonenzymatic glycation and oxidation of amino groups on proteins to form Amadori adducts. Over time, the adducts undergo additional rearrangements, dehydrations, and cross-linking with other proteins to form complexes known as Advanced Glycosylation End Products (AGEs). Factors which promote formation of AGEs included delayed protein turnover (*e.g.* as in amyloidoses), accumulation of macromolecules having high lysine content, and high blood glucose levels 20 (*e.g.* as in diabetes) (Hori *et al.*, *J. Biol. Chem.* 270: 25752-761, (1995)). AGEs have been implicated in a variety of disorders including complications associated with diabetes and normal aging.

25 AGEs display specific and saturable binding to cell surface receptors on monocytes, macrophages, endothelial cells of the microvasculature, smooth muscle cells, mesengial cells, and neurons. The Receptor for Advanced Glycated Endproducts (RAGE) is a member of the immunoglobulin super family of cell surface molecules. The extracellular (N-terminal) domain of RAGE includes three immunoglobulin-type regions: one V (variable) type domain followed by two C-type (constant) domains (Neerper *et al.*, *J. Biol. Chem.*, 267:14998-15004 30 (1992); Schmidt *et al.*, *Circ. (Suppl.)* 96#194 (1997)). A single transmembrane spanning domain and a short, highly charged cytosolic tail follow the extracellular domain. The N-terminal, extracellular domain can be isolated by proteolysis of RAGE to generate soluble RAGE (sRAGE) comprised of the V and C domains.

RAGE is expressed in most tissues, and in particular, is found in cortical neurons during embryogenesis (Hori *et al.*, *J. Biol. Chem.*, 270:25752-761 (1995)). Increased levels

of RAGE are also found in aging tissues (Schleicher *et al.*, *J. Clin. Invest.*, **99** (3): 457-468 (1997)), and the diabetic retina, vasculature and kidney (Schmidt *et al.*, *Nature Med.*, **1**:1002-1004 (1995)). Activation of RAGE in different tissues and organs leads to a number of pathophysiological consequences. RAGE has been implicated in a variety of conditions
5 including: acute and chronic inflammation (Hofmann *et al.*, *Cell* **97**:889-901 (1999)) the development of diabetic late complications such as increased vascular permeability (Wautier *et al.*, *J. Clin. Invest.*, **97**:238-243 (1995), nephropathy (Teillet *et al.*, *J. Am. Soc. Nephrol.*, **11**:1488-1497 (2000), atherosclerosis (Vlassara *et. al.*, *The Finnish Medical Society DUODECIM, Ann. Med.*, **28**:419-426 (1996), and retinopathy (Hammes *et al.*, *Diabetologia*, **42**:603-607 (1999)). RAGE has also been implicated in Alzheimer's disease (Yan *et al.*, *Nature*, **382**:685-691 (1996)); erectile dysfunction; and in tumor invasion and metastasis (Taguchi *et al.*, *Nature*, **405**:354-357 (2000)).

In addition to AGEs, other compounds can bind to, and modulate RAGE. In normal development, RAGE interacts with amphotericin, a polypeptide which mediates neurite
15 outgrowth in cultured embryonic neurons (Hori *et al.*, 1995). RAGE has also been shown to interact with calgranulin-like ligands and β -amyloid (Yan *et al.*, *Nature*, **389**:589-595, (1997); Yan *et al.*, *Nature*, **382**:685-691 (1996); Yan *et al.*, *Proc. Natl. Acad. Sci.*, **94**:5296-5301 (1997)).

Binding of ligands such as AGEs, S100/calgranulin, β -amyloid, CML (N^{ϵ} -Carboxymethyl lysine), and amphotericin to RAGE has been shown to modify expression of a variety of genes. For example, in many cell types interaction between RAGE and its ligands generates oxidative stress, which thereby results in activation of the free radical sensitive transcription factor NF- κ B, and the activation of NF- κ B regulated genes, such as the cytokines IL-1 β , TNF- α , and the like. In addition, several other regulatory pathways, such as those involving p21ras, MAP kinases, ERK1 and ERK2, have been shown to be activated by binding of AGEs and other ligands to RAGE. In fact, transcription of RAGE itself is regulated at least in part by NF- κ B. Thus, an ascending, and often detrimental, spiral is fueled by a positive feedback loop initiated by ligand binding. Antagonizing binding of physiological ligands to RAGE, therefore, is a logical target for down-regulation of the pathophysiological changes brought about by excessive concentrations of AGEs and other ligands for RAGE.
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Thus, there is a need for the methods to discover compounds which modulate binding of physiological ligands to the RAGE receptor. The method should comprise an assay which

is sensitive and yet highly specific. Preferably, the method should utilize RAGE, or the binding site of RAGE itself, thereby allowing the development of modulators which vary in structure and physiological effect. Ideally, the method should lend itself to high throughput, automative techniques suitable for large scale screening of multiple compounds.

5

SUMMARY

The present invention relates to the use of a high throughput assay for the discovery of compound that modulate RAGE. In one aspect, the present invention comprises a method for detection of RAGE modulators comprising: (a) adsorbing a RAGE ligand onto a solid surface; (b) adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof to the preadsorbed ligand; (c) adding an anti-RAGE antibody which binds to the RAGE protein or fragment thereof; (d) determining the amount of RAGE protein or fragment thereof bound to the ligand by measuring the amount of anti-RAGE antibody bound to the solid surface; and (e) comparing the amount of RAGE protein or fragment thereof bound to the ligand in the presence of varying amounts of the compound of interest.

In another aspect, the present invention comprises a method for detection of RAGE modulators comprising: (a) adsorbing a RAGE ligand onto a solid surface; (b) adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof to the preadsorbed ligand; (c) adding an anti-RAGE antibody which binds to the RAGE protein or fragment thereof and a secondary antibody which binds to the anti-RAGE antibody; (d) measuring the secondary antibody bound to the anti-RAGE antibody; and (e) comparing the amount of RAGE bound to the ligand in the presence of varying amounts of the compound of interest.

25

In another aspect, the invention comprises compounds identified by the methods of the invention.

In yet another aspect, the invention comprises a kit for detection of RAGE modulators comprising: (a) a solid surface comprising a RAGE ligand; (b) a separately packaged solution comprising a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof; (c) a detection system comprising at least one agent which specifically binds to the RAGE protein or fragment thereof; (d) separately packaged reagents for the detection system; and (e) instructions for use.

The foregoing focuses on the more important features of the invention in order that the detailed description which follows may be better understood and in order that the present

contribution to the art may be better appreciated. There are, of course, additional features of the invention which will be described hereinafter and which will form the subject matter of the claims appended hereto. It is to be understood that the invention is not limited in its application to the details set forth in the following description and drawings. The invention is capable of other embodiments and of being practiced or carried out in various ways.

From the foregoing summary, it is apparent that an object of the present invention is to provide a rapid, high-throughput method for the detection of RAGE modulators. These, together with other objects of the present invention, along with the various features of novelty which characterize the invention, are pointed out with particularity in the claims annexed to and forming a part of this document.

BRIEF DESCRIPTION OF THE DRAWINGS

Various features, aspects and advantages of the present invention will become more apparent with reference to the following description, appended claims, and accompanying drawings.

Figure 1 shows a schematic representation of an embodiment of the method of the present invention.

Figure 2 shows an aspect of an embodiment of the present invention comprising: (A) SEQ ID NO: 1, the amino acid sequence for human RAGE as reported in GenBank/EMBL database, accession number XM004205; (B) SEQ ID NO: 2, the amino acid sequence of human sRAGE; (C) SEQ ID NO: 3, the amino acid sequence of the V-domain of human RAGE; and (D) SEQ ID NO: 4, the N-terminal fragment of the V-domain of human RAGE.

Figure 3 shows an aspect of an embodiment of the present invention comprising optimization of S-100b concentration where sRAGE was present at 1.0×10^{-4} mg/ml, polyclonal antibody to sRAGE was present at 3.0×10^{-3} mg/ml, and overnight incubations were done using S-100b at concentrations which ranged from about 2 ng/well to 4 mg/well.

Figure 4 shows an aspect of an embodiment of the present invention comprising optimization of sRAGE concentration where 500 ng/well S-100b was used to coat wells, and polyclonal antibody to sRAGE was present at 3.0×10^{-3} mg/ml, and sRAGE concentration ranged from about 2 to 10 nM.

Figure 5 shows an aspect of an embodiment of the present invention comprising optimization of antibody concentration where sRAGE was present at 1.0×10^{-4} mg/ml, incubations were done using S-100b at 50, 500, 1,000 and 2,000 ng/well as indicated, and polyclonal antibody concentration ranged from 5×10^{-6} to 0.054 mg/ml.

Figure 6 shows an aspect of an embodiment of the present invention comprising optimization of DMSO tolerance where 500 ng/well S-100b was used to coat wells, and polyclonal or monoclonal antibody to sRAGE was present at 3.0×10^3 mg/ml and 1.9×10^4 mg/ml respectively, and the concentration of sRAGE was 2.2×10^4 mg/ml.

5 Figure 7 shows an aspect of an embodiment of the present invention comprising the variation in sample OD for 88 samples as a function of individual well position, where the average OD comprised 0.705, the standard deviation (SD) is 0.054, and the 95% confidence value (CV) is 7.73%.

10 Figure 8 shows an aspect of an embodiment of the present invention comprising a saturation curve for sRAGE binding to S-100b, where 50 ng/well S-100b was used to coat the wells, polyclonal antibody to sRAGE was present at 3.0×10^3 mg/ml, and sRAGE concentration ranged from about 0.5 to 9 nM.

15 Figure 9 shows an aspect of an embodiment of the present invention comprising a saturation curve for sRAGE binding to S-100b, where 100 ng/well S-100b was used to coat the wells, polyclonal antibody to sRAGE was present at 3.0×10^3 mg/ml, and sRAGE concentration ranged from about 0.3 to 10 nM.

20 Figure 10 shows an aspect of an embodiment of the present invention comprising a saturation curve for sRAGE binding to S-100b, where 500 ng/well S-100b was used to coat the wells, and polyclonal antibody to sRAGE was present at 3.0×10^3 mg/ml, and sRAGE concentration ranged from about 24 to 470 nM.

Figure 11 shows an aspect of an embodiment of the present invention comprising a saturation curve for sRAGE binding to S-100b, where 100 ng/well S-100b was used to coat the wells, and polyclonal antibody to sRAGE was present at 3.0×10^3 mg/ml, and sRAGE concentration ranged from about 5 to 76 nM.

25 Figure 12 shows an aspect of an embodiment of the present invention comprising inhibition of sRAGE binding by Compound 1.

Figure 13 shows an aspect of an embodiment of the present invention comprising the structure of Compound 1.

30 Figure 14 shows an aspect of an embodiment of the present invention comprising an inhibition of sRAGE activation of the transcription factor NF- κ B by Compound 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of a high throughput assay for the discovery of compounds that modulate RAGE. The method measures binding of ligands to RAGE, or fragments of RAGE, under conditions in which the physiological and structural integrity of the receptor is maintained. Those compounds which bind are assessed for physiological activity.

In one aspect, the present invention comprises a method for detection of RAGE modulators comprising: (a) adsorbing a RAGE ligand onto a solid surface; (b) adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof to the preadsorbed ligand; (c) adding anti-RAGE antibody which binds to the RAGE protein or fragment thereof; and (d) determining the amount of RAGE protein or fragment thereof bound to the ligand by measuring the amount of anti-RAGE antibody bound to the solid surface; and (e) comparing the amount of RAGE protein or fragment thereof bound to the ligand in the presence of varying amounts of the compound of interest.

In an embodiment, the fragment of RAGE is the soluble, extracellular portion of RAGE (sRAGE), as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto. In another embodiment, the fragment of RAGE is the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto. In yet another embodiment, the fragment of RAGE is a fragment of the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto. Preferably, the method comprises measuring the ability of the compound of interest to modulate the ability of RAGE, or protein substantially homologous thereto, to activate cellular processes. More preferably, agonists comprise stimulation of RAGE-activated cellular processes and antagonists comprise inhibition of RAGE-activated cellular processes. Even more preferably, the cellular process comprises activation of NF- κ B gene transcription.

In an embodiment, the antibody to RAGE comprises a monoclonal antibody. In an embodiment, the antibody to RAGE comprises polyclonal antibody.

Preferably, the method further comprises addition of a second antibody which recognizes the anti-RAGE antibody. More preferably, the anti-RAGE antibody and the secondary antibody are allowed to complex prior to being added. Also preferably, the method further comprises a colorimetric assay for the secondary antibody.

In an embodiment, the solid surface comprises a reaction vessel. In another embodiment, the solid surface comprises a dip-stick.

Preferably, the ligand comprises an advanced glycated endproduct, or fragment thereof. Also preferably, the ligand comprises carboxymethyl-lycine-modified AGE. Also preferably, the ligand comprises β -amyloid. Also preferably, the ligand comprises calgranulin. Also preferably, the ligand comprises S-100b. Also preferably, the ligand comprises amphoterin.

5 Preferably, the compound of interest comprises a peptide. Also preferably, the compound of interest comprises a peptidomimetic. Also preferably, the compound of interest comprises an organic compound. Also preferably, the compound of interest comprises an inorganic compound. Also preferably, the compound of interest comprises a lipid. Also
10 preferably, the compound of interest comprises a carbohydrate. Also preferably, the compound of interest comprises nucleic acid.

The invention relates to the use of a physiologically relevant binding assay to discover compounds identified which modulate RAGE. In one aspect, the invention comprises compounds identified by a method having steps comprising: (a) adsorbing a RAGE ligand onto a solid surface; (b) adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof to the preadsorbed ligand; (c) adding anti-RAGE antibody which binds to RAGE protein or a fragment thereof; (d) determining the amount of RAGE protein or a fragment thereof bound to the ligand by measuring the amount of anti-RAGE antibody bound to the solid surface; and (e) comparing the amount of RAGE protein or a fragment thereof bound to the ligand in the presence of varying amounts of the compound of interest.

In an embodiment, the fragment of RAGE is the soluble, extracellular portion of RAGE (sRAGE), as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto. In another embodiment, the fragment of RAGE is the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto. In yet another embodiment, the fragment of RAGE is a fragment of the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.

25 Preferably, the method of identifying the compounds further comprises measuring the ability of the compound of interest to modulate the ability of RAGE comprising SEQ ID NO: 1 or a protein substantially homologous thereto, to activate cellular processes. Preferably, the compound identified by the methods of the invention is used to treat symptoms of diabetes and symptoms of diabetic late complications. Also preferably, the compound identified by the methods of the invention is used to treat amyloidoses. Also preferably, the compound

identified by the methods of the invention is used to treat Alzheimer's disease. Also preferably, the compound identified by the methods of the invention is used to treat cancer. Also preferably, the compound identified by the methods of the invention is used to treat inflammation. Also preferably, the compound identified by the methods of the invention is used to treat kidney failure. Also preferably, the compound identified by the methods of the invention is used to treat systemic lupus nephritis or inflammatory lupus nephritis. Also preferably, the compound identified by the methods of the invention is used to treat erectile dysfunction.

The methods of the invention provide a flexible protocol which is adapted to a variety of reagents without reduction in reliability or precision. Thus, a variety of developing reagents or antibody preparations may be used. In one aspect, the present invention comprises a method for detection of RAGE modulators comprising: (a) adsorbing a RAGE ligand onto a solid surface; (b) adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof to the preadsorbed ligand; (c) adding anti-RAGE antibody which binds to the RAGE protein or a fragment thereof and a secondary antibody which binds to the anti-RAGE antibody; (d) measuring the secondary antibody bound to the anti-RAGE antibody; (e) comparing the amount of RAGE or fragment thereof bound to the ligand in the presence of varying amounts of the compound of interest.

In an embodiment, the fragment of RAGE is the soluble, extracellular portion of RAGE (sRAGE)), as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto. In another embodiment, the fragment of RAGE is the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto. In yet another embodiment, the fragment of RAGE is a fragment of the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.

Preferably, the method comprises measuring the ability of the compound of interest to modulate the ability of RAGE, or protein substantially homologous thereto, to activate cellular processes. More preferably, agonists comprise stimulation of RAGE-activated cellular processes and antagonists comprise inhibition of RAGE-activated cellular processes. Even more preferably, the cellular process comprise activation of NF- κ B gene transcription.

Preferably, the method further comprises a colorimetric assay for the secondary antibody. In an embodiment, the anti-RAGE antibody and secondary antibody are allowed to complex prior to being added to the reaction vessel comprising RAGE, thus maintaining the RAGE binding domain in an non-antibody bound (*i.e.* unperturbed) state. In an embodiment,

the solid surface comprises a reaction vessel. In an embodiment, the solid surface comprises a dip-stick.

In yet another aspect, the invention comprises a kit for detection of RAGE modulators comprising: (a) a solid surface comprising a RAGE ligand; (b) a separate packaged solution comprising a protein comprising RAGE comprising SEQ ID NO: 1 or fragment thereof; (c) a detection system comprising at least one agent which specifically binds to the RAGE, or fragment thereof; (c) separately packaged colorimetric reagents for the detection system; and (d) instructions for use. In an embodiment, the kit comprises a separately packaged antibody which binds to RAGE, or fragment thereof. In an embodiment, the fragment of RAGE is the soluble, extracellular portion of RAGE (sRAGE)), as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto. In an embodiment, the fragment of RAGE is the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto. In yet another embodiment, the fragment of RAGE is a fragment of the V-domain of RAGE, as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.

The present invention comprises a method for the detection of RAGE modulators which utilizes ligand and RAGE, or a fragments of RAGE, which have not been chemically modified. Thus, the method of the present invention is able to measure binding of ligands and the modification of such binding, with physiological specificity. The method is more reliable than assay systems which use radiolabelled binding partners, and is highly sensitive due to the use of an enzyme coupled development system. In addition, the assay employs non-toxic reagents which do not present a threat to laboratory personnel or the environment.

Thus, in one aspect, the invention comprises the use of ELISA type assays as a method for the detection of RAGE modulators. In an embodiment, and referring now to Figure 1, a RAGE ligand, such as S-100b, is allowed to adsorb to the wells of a microtiter plate. The wells are then washed using buffered saline to remove the unbound ligand, and sites which comprise non-specific protein binding sites are blocked using blocking buffer containing bovine serum albumin (BSA) or another protein unrelated to RAGE binding. The blocking buffer is then removed, as for example by aspiration, and the wells washed several times to remove traces of blocking buffer. A solution comprising the compound of interest (*i.e.* a putative RAGE modulator) and RAGE comprising SEQ ID NO: 1, or fragment thereof, is added to each well. For example, in an embodiment, the RAGE fragment used is sRAGE (Taguchi *et al.*, *Nature*, 405:354-360 (1996)). The mixture is incubated under conditions

which allow physiological binding of the RAGE protein (*e.g.* sRAGE) to the immobilized ligand in each well.

Preferably, during the binding reaction of sRAGE to the immobilized ligand, a complex comprising: (a) anti-RAGE antibody (anti-RAGE); (b) biotinylated goat anti-mouse IgG (B-anti-IgG); and (c) streptavidin labeled alkaline phosphatase (S-AP), is allowed to form in a separate reaction vessel. After a suitable time for binding of sRAGE to the immobilized ligand, the reaction wells are washed to remove traces of unbound sRAGE and the compound of interest, and the anti-RAGE: B-anti-IgG: S-AP complex added to each well. The addition of the complex allows for immobilization of the alkaline phosphatase enzyme via interaction of the complex with sRAGE immobilized in the well (Figure 1). By adding the immune complex separately, the sRAGE is allowed to bind to the ligand and compound of interest (*i.e.* putative modulator) without interference due to antibody binding. The amount of alkaline phosphatase, and thus the amount of immobilized sRAGE, can then be detected using a colorimetric assay for conversion of *para*-nitrophenyl phosphate (pNPP) to *para*-nitrophenol (pNP).

As used herein, RAGE encompasses a peptide which has the full amino acid sequence of RAGE as shown in Figure 2A (SEQ ID NO: 1) or a portion of that amino acid sequence (Neeper *et al.*, (1992)). The binding domain of RAGE comprises that region of the protein which is able to bind ligands with physiological specificity. A fragment of RAGE is at least 5 amino acids in length, but more preferably greater than 30 amino acids in length, but is substantially less than the full amino acid sequence. Thus, in another embodiment, the fragment of RAGE comprises sRAGE (SEQ ID NO: 2; Figure 2B), wherein sRAGE is the RAGE protein free from the cell membrane (Park *et al.*, *Nature Med.*, 4:1025-1031 (1998)). In another embodiment, the RAGE or fragment thereof comprises the V domain (SEQ ID NO: 3; Figure 2C) (Neeper *et al.*, (1992); Schmidt *et al.*, (1997)), or a fragment thereof (SEQ ID NO: 4, Figure 2D). In yet another embodiment, the RAGE or fragment thereof is a synthetic peptide.

The terms "substantially homologous" when referring to polypeptides refer to at least two amino acid sequences which when optimally aligned, are at least 75% homologous, preferably at least about 85% homologous, more preferably at least about 90% homologous, and still more preferably 95% homologous. Optimal alignment of sequences for aligning a comparison may be conducted using the algorithms standard in the art (*e.g.* Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981); Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970); Pearson and Lipman, *Proc. Natl. Acad. Sci. (USA)*, 85:2444 (1988)) or by

computerized versions of these algorithms (Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive, Madison, WI).

Preferably, the concentration of ligand used for coating the solid surface (*i.e.* reaction wells) allows any antagonism of binding of the immobilized ligand to sRAGE to be detected.

5 For example, referring now to Figure 3, where sRAGE comprises 0.1 µg/ml, and anti-sRAGE antibody comprises 3.0 µg/ml, almost maximum signal is seen with 500 ng S-100b, and ligand concentrations between 10 to 500 ng/well comprise a linear increase in sRAGE binding.

Also preferably, and referring now to Figure 4, the assay is designed to allow 10 selection of an amount of sRAGE which comprises a linear region of binding of sRAGE and the immobilized ligand. For example, if too low of a concentration of sRAGE is employed, the detection of binding will be difficult due to low signal. If excess sRAGE is used, however, the ability of compound of interest to compete with the immobilized ligand will be lessened due to the excess sRAGE binding to both the ligand and the compound being tested.

15 Also preferably, the assay is designed to allow optimization of the anti-RAGE antibody. Thus, in an embodiment, and referring now to Figure 5, the amount of antibody used can be titered to allow for maximal signal, but not so high as to comprise high levels of background binding. More preferably, the amount of antibody allowing for maximum signal comprises a similar range of antibody regardless of the concentration of immobilized ligand.

20 Also preferably, the assay is optimized to be independent of solvents required for dissolving the compounds being tested. In an embodiment, and referring to Figure 6, for dimethyl sulfoxide (DMSO) concentrations of 2%, which is the concentration of DMSO generally employed for dissolution of organic compounds of interest in the assay, the assay provides maximal signal. Even at concentrations as high as 10% DMSO, the assay provides 25 almost maximal signal.

Thus, the assay allows for optimization of each component, such that variation of assay results due to environmental factors and experimental variation is minimized.

30 Preferably, once optimized for a specific ligand, sRAGE concentration, and anti-sRAGE antibody concentration, the assay will provide a reliable assessment of sample binding affinity regardless of the specific competing compound (*i.e.* putative modulator) which is being tested. Also preferably, the assay comprises a high throughput assay that is reproducible and precise. Referring now to Figure 7, in an embodiment the assay comprises a variance of less than 10%.

Without being bound to any particular theory, a premise of the assay is that binding of sRAGE to the ligand immobilized in the assay well will reflect the nature of the binding of RAGE to the ligand *in vivo*. Thus, in an embodiment, the immobilized ligand will bind RAGE comprising SEQ ID NO: 1, or fragment thereof, in a specific and saturable manner.

5 For example, and referring now to Figures 8-11, sRAGE at concentrations ranging from about 0.3 to 480 nM binds in a saturable manner to S-100b ligand immobilized in a microtiter well. In addition, an analysis of the data by means of Scatchard transformation shows both high and low affinity sites with dissociation constants (Kd) similar to the Kd for RAGE reported previously (Hofmann *et al.*, *Cell*, 97:889-901 (1999); Hori *et al.*, *J. Biol. Chem.*, 10 270:25752-25761 (1995)).

Preferably, the assay of the present invention is able to detect compounds that antagonize both low and high affinity binding sites. Referring now to Figures 8 and 9, using sRAGE at concentrations ranging from 0.3 to 10 nM, a high affinity binding site comprising a Kd of about 2-6 nM is detected. Also, referring now to Figures 10 and 11, using sRAGE at 15 concentrations ranging from 24 to 480 nM, and 5 to 80 nM respectively, a lower affinity binding site comprising a Kd of about 60 nM is detected.

In an embodiment, the anti-RAGE antibody is polyclonal. Polyclonal antibodies are a heterogeneous population of antibody molecules derived from the sera of animals immunized with the antigen of interest. Adjuvants such as Freund's (complete and incomplete), peptides, 20 oil emulsions, lysolecithin, polyols, polyanions and the like may be used to increase the immune response. Thus, in an embodiment, polyclonal antibody to sRAGE is prepared by injection of sRAGE supplemented with an adjuvant into rabbits using methods known in the art (*e.g.* Schmidt *et al.*, *J. Biol. Chem.*, 267:14987-14997 (1992)).

Monoclonal antibodies are homogeneous populations of antibodies to a particular 25 antigen, and are generally obtained by any technique which provides for production of antibody by continuous cell lines in culture (*see e.g.* U.S. Patent No. 4,873,313). For example, in an embodiment sRAGE protein is used for production of monoclonal antibodies using methods known in the art (Zymed Laboratories, San Francisco, CA).

Again without being bound to any particular theory, a premise of the assay is that one 30 of the components in the detection system labeled. It is contemplated that the detection system may use reagents which are can be seen visually, and which are thereby defined as "colorimetric.. For example, in an embodiment, a complex of anti-RAGE antibody: biotinylated goat anti-human IgG: streptavidin labeled alkaline phosphatase is used to detect sRAGE bound to the ligand. The alkaline phosphatase enzyme catalyses the conversion of

light yellow *para*-nitrophenyl phosphate (pNPP) to the pink product *para*-nitrophenol. It is, however, within the scope of the present invention to use variations of the detection system which are known to those in the art. For example, in a embodiment, the secondary antibody which binds to anti-RAGE antibody is labeled with streptavidin and then detected with 5 biotinylated alkaline phosphatase. Other labeling moieties include fluorescein, digoxigenin, 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt, and the like. In another embodiment, streptavidin-labeled horseradish peroxidase is employed as the detection system. Alternatively, in another embodiment, a third antibody comprising biotinylated anti-goat IgG, can be used to detect a non-biotinylated anti-mouse IgG. In yet 10 another embodiment, the anti-RAGE antibody itself may be biotinylated, and detected using streptavidin-labeled alkaline phosphatase.

The assay may be carried out with the ligand immobilized to the surface of a reaction vessel or other solid surface. For example, in an embodiment, the ligand is immobilized in a microtiter well. In another embodiment, the ligand is immobilized on a dip-stick. In this 15 format, the assay may be performed by transferring the dipstick comprising an immobilized ligand first to solution comprising sRAGE and a test compound (or biological sample), and then to a reagent for detecting sRAGE binding to the dipstick (e.g. an anti-sRAGE antibody:biotinylated IgG: streptavidin-alkaline phosphatase complex). A reduction in signal on the dipstick as compared to a known control would indicate the sample comprises a 20 compound which can bind to RAGE. In an embodiment, the assay can detect increased RAGE ligands in a biological sample. Preferably, such ligands include AGEs, amphotericin, (Hori *et al.*, 1995), and β -amyloid (Yan *et al.*, *Nature* 389:589-595, (1997); Yan *et al.*, *Nature* 382:685-691 (1996); Yan *et al.*, *Proc. Natl. Acad. Sci.*, 94:5296-5301 (1997)). In another embodiment, the ligand is immobilized on beads, a nylon membrane, or any other solid 25 support.

The methods of the invention provide binding assays to identify compounds that interact with RAGE under physiological binding conditions. In this respect, physiological binding conditions comprise those conditions which result in binding affinities similar to those seen *in vivo*. Thus, in another aspect, the present invention comprises the compounds 30 identified by the methods of the invention. Compounds identified by the methods of the invention may comprise several different chemical types. For example, in an embodiment the compound is a peptide. In another embodiment, the compound is a peptidomimetic. In another embodiment, the compound is an organic molecule. In another embodiment, the

compound is an inorganic molecule. In some cases, the compound of interest may be derivatized to increase half-life.

The compounds of the present invention may be a peptidomimetic which is at least partly unnatural. Preferably the compound of interest may be modified to increase stability, 5 efficacy, potency and bioavailability. The compound may be synthetically prepared. The compound may include L-, D-, or unnatural amino acids, alpha-disubstituted amino acids, N-alkyl amino acids, or lactic acid. In an embodiment, the compound comprises a peptidomimetic having a peptide backbone or amino acid replaced with a suitable mimetic.

For example, as shown in Figure 12, Compound 1, the structure of which is shown in 10 Figure 13, inhibits sRAGE binding to S-100b with $IC_{50} = 1.8 \pm 0.2 \mu\text{M}$, wherein IC_{50} is defined as the concentration of the agent (*i.e.* Compound 1) which comprises 50% inhibition of sRAGE binding.

In an embodiment, compounds identified by the binding assay are further tested as 15 having the ability to modulate a biological activity of RAGE comprising SEQ ID NO: 1, or fragment thereof. For example, compounds can be tested for their ability to modulate RAGE induced increases in gene expression. Thus, in an embodiment and referring now to Figure 14, compounds identified by the binding assay are used to modulate RAGE activation of NF- κ B mediated transcription of a reporter gene. Preferably, compounds which modulate ligand binding to RAGE comprising SEQ ID NO: 1, or fragment thereof, will be identified as 20 modulating the effects of RAGE on other cellular processes.

In an embodiment, the invention comprises treatment of human disease by 25 compounds identified by the methods of the invention. Preferably, compounds identified by the methods of the invention are used to treat diabetes. It has been shown that nonenzymatic glycoxidation of macromolecules ultimately resulting in the formation of advanced glycation endproducts (AGEs) is enhanced at sites of inflammation, in renal failure, in the presence of hyperglycemia and other conditions associated with systemic or local oxidant stress (Dyer *et al.*, *J. Clin. Invest.*, 91:2463-2469 (1993); Reddy *et al.*, *Biochem.*, 34:10872-10878 (1995); Dyer *et al.*, *J. Biol. Chem.*, 266:11654-11660 (1991); Degenhardt *et al.*, *Cell Mol. Biol.*, 44:1139-1145 (1998)). Accumulation of AGEs in the vasculature can occur focally, as in the 30 joint amyloid composed of AGE- β_2 -microglobulin found in patients with dialysis-related amyloidosis (Miyata *et al.*, *J. Clin. Invest.*, 92:1243-1252 (1993); Miyata *et al.*, *J. Clin. Invest.*, 98:1088-1094 (1996)), or generally, as exemplified by the vasculature and tissues of patients with diabetes (Schmidt *et al.*, *Nature Med.*, 1:1002-1004 (1995)). The progressive

accumulation of AGEs over time in patients with diabetes suggests that endogenous clearance mechanisms are not able to function effectively at sites of AGE deposition. Such accumulated AGEs have the capacity to alter cellular properties by a number of mechanisms. Although RAGE is expressed at low levels in normal tissues and vasculature, in an
5 environment where the receptor's ligands accumulate, it has been shown that RAGE becomes upregulated (Li *et al.*, *J. Biol. Chem.*, **272**:16498-16506 (1997); Li *et al.*, *J. Biol. Chem.*, **273**:30870-30878 (1998); Tanaka *et al.*, *J. Biol. Chem.*, **275**:25781-25790(2000)). RAGE expression is increased in endothelium, smooth muscle cells and infiltrating mononuclear phagocytes in diabetic vasculature. Also, studies in cell culture have demonstrated that AGE
10 RAGE interaction caused changes in cellular properties important in vascular homeostasis.

Also preferably, compounds identified by the methods of the invention are used to treat atherosclerosis. Thus, it has been shown that ischemic heart disease is particularly high in patients with diabetes (Robertson, *et al.*, *Lab Invest.*, **18**:538-551 (1968); Kannel *et al.*, *J. Am. Med. Assoc.*, **241**:2035-2038 (1979); Kannel *et al.*, *Diab. Care*, **2**:120-126 (1979)). In
15 addition, studies have shown that atherosclerosis in patients with diabetes is more accelerated and extensive than in patients not suffering from diabetes (see e.g. Waller *et al.*, *Am. J. Med.*, **69**:498-506 (1980); Crall *et al.*, *Am. J. Med.* **64**:221-230 (1978); Hamby *et al.*, *Chest*, **2**:251-257 (1976); and Pyorala *et al.*, *Diab. Metab. Rev.*, **3**:463-524 (1978)). Although the reasons for accelerated atherosclerosis in the setting of diabetes are many, it has been shown that
20 reduction of AGEs can reduce plaque formation.

Also preferably, compounds identified by the methods of the invention are used to treat amyloidoses and Alzheimer's disease. It has been shown that RAGE binds β -sheet fibrillar material regardless of the composition of the subunits (amyloid- β peptide, amylin, serum amyloid A, prion-derived peptide) (Yan *et al.*, *Nature*, **382**:685-691 (1996); Yan *et al.*, *Nat. Med.*, **6**:643-651 (2000)). In addition, deposition of amyloid has been shown to result in enhanced expression of RAGE. For example, in the brains of patients with Alzheimer's disease (AD), RAGE expression increases in neurons and glia (Yan, *et al.*, *Nature* **382**:685-691 (1996)). The consequences of β -amyloid peptide (A β) interaction with RAGE appear to be quite different on neurons versus microglia. Whereas microglia become activated as a
25 consequence of A β -RAGE interaction, as reflected by increased motility and expression of cytokines, early RAGE-mediated neuronal activation is superceded by cytotoxicity at later times. Further evidence of a role for RAGE in cellular interactions of A β is shown by the inhibition of A β -induced cerebral vasoconstriction and transfer of the peptide across the blood-brain barrier to brain parenchyma when the receptor was blocked (Kumar *et al.*,

Neurosci. Program, p141-#275.19 (2000)). Inhibition of RAGE-amyloid interaction has been shown to decrease expression of cellular RAGE and cell stress markers (as well as NF- κ B activation), and diminish amyloid deposition (Yan *et al.*, *Nat. Med.*, 6:643-651 (2000)) suggesting a role for RAGE-amyloid interaction in both perturbation of cellular properties in 5 an environment enriched for amyloid (even at early stages) as well as in amyloid accumulation.

Also preferably, compounds identified by the methods of the invention are used to treat cancer. For example, amphoterin is a high mobility group I nonhistone chromosomal DNA binding protein (Rauvala *et al.*, *J. Biol. Chem.*, 262:16625-16635 (1987); Parkkinen *et al.*, *J. Biol. Chem.* 268:19726-19738 (1993)) which has been shown to interact with RAGE. It has been shown that amphoterin promotes neurite outgrowth, as well as serving as a surface for assembly of protease complexes in the fibrinolytic system (also known to contribute to cell mobility). In addition, a local tumor growth inhibitory effect of blocking RAGE has been observed in a primary tumor model (C6 glioma), the Lewis lung metastasis model 10 (Taguchi *et al.*, *Nature* 405:354-360 (2000)), and spontaneously arising papillomas in mice expressing the v-Ha-ras transgene (Leder *et al.*, *Proc. Natl. Acad. Sci.*, 87:9178-9182 15 (1990)).

Also preferably, compounds identified by the methods of the invention are used to treat inflammation. Also preferably, the compounds identified by the methods of the 20 invention are used to treat kidney failure. Also preferably, the compounds identified by the methods of the invention are used to treat systemic lupus nephritis or inflammatory lupus nephritis. For example, the S100/calgranulins have been shown to comprise a family of closely related calcium-binding polypeptides characterized by two EF-hand regions linked by a connecting peptide (Schafer *et al.*, *TIBS*, 21:134-140 (1996); Zimmer *et al.*, *Brain Res. Bull.*, 37:417-429 (1995); Rammes *et al.*, *J. Biol. Chem.*, 272:9496-9502 (1997); Lugering *et al.*, *Eur. J. Clin. Invest.*, 25:659-664 (1995)). Although they lack signal peptides, it has long 25 been known that S100/calgranulins gain access to the extracellular space, especially at sites of chronic immune/inflammatory responses, as in cystic fibrosis and rheumatoid arthritis. RAGE is a receptor for many members of the S100/calgranulin family, mediating their proinflammatory effects on cells such as lymphocytes and mononuclear phagocytes. Also, studies on delayed-type hypersensitivity response, colitis in IL-10 null mice, collagen-induced arthritis, and experimental autoimmune encephalitis models suggest that RAGE-ligand interaction (presumably with S-100/calgranulins) has a proximal role in the 30 inflammatory cascade.

Also preferably, compounds identified by the methods of the invention are used to treat erectile dysfunction. Relaxation of the smooth muscle cells in the cavernosal arterioles and sinuses results in increased blood flow into the penis, raising corpus cavernosum pressure to culminate in penile erection. Nitric oxide is considered the principle stimulator of 5 cavernosal smooth muscle relaxation (Wingard *et al*, *Nature Medicine*, Jan;7(1):119-122 (2001)). RAGE activation produces oxidants (Yan *et al*, *J. Biol. Chem.*, 269:9889-9887, 1994) via an NADH oxidase-like enzyme, therefore suppressing the circulation of nitric 10 oxide. Potentially by inhibiting the activation of RAGE signaling pathways by decreasing the intracellular production of AGEs, generation of oxidants will be attenuated. Rage blockers may promote and facilitate penile erection by blocking the access of ligands to RAGE. The 15 calcium-sensitizing Rho-kinase pathway may play a synergistic role in cavernosal vasoconstriction to maintain penile flaccidity. The antagonism of Rho-kinase results in increased corpus cavernosum pressure, initiating the erectile response independently of nitric oxide (Wingard *et al.*, 2001). One of the signaling mechanisms activated by RAGE involves the Rho-kinase family such as cdc42 and rac (Huttunen *et al*, *J Biol Chem.*, 274:19919-24 1999)). Thus, inhibiting activation of Rho-kinases via suppression of RAGE signaling pathways will enhance and stimulate penile erection independently of nitric oxide.

In one aspect, the present invention also provides a method for inhibiting the interaction of an AGE with RAGE in a subject which comprises administering to the subject 20 a therapeutically effective amount of a compound identified by the methods of the invention. A therapeutically effective amount is an amount which is capable of preventing interaction of AGE/RAGE in a subject. Accordingly, the amount will vary with the subject being treated. Administration of the compound may be hourly, daily, weekly, monthly, yearly or a single 25 event. Preferably, the effective amount of the compound comprises from about 1 ng/kg body weight to about 100 mg/kg body weight. More preferably, the effective amount of the compound comprises from about 1 µg/kg body weight to about 50 mg/kg body weight. Even more preferably, the effective amount of the compound comprises from about 10 µg/kg body weight to about 10 mg/kg body weight. The actual effective amount will be established by dose/response assays using methods standard in the art (Johnson *et al.*, *Diabetes*. 42: 1179, 30 (1993)). Thus, as is known to those in the art, the effective amount will depend on bioavailability, bioactivity, and biodegradability of the compound.

In an embodiment, the subject is an animal. In an embodiment, the subject is a human. In an embodiment, the subject is suffering from an AGE-related disease such as diabetes, amyloidoses, renal failure, aging, or inflammation. In another embodiment, the

subject comprises an individual with Alzheimer's disease. In an alternative embodiment, the subject comprises an individual with cancer. In yet another embodiment, the subject comprises an individual with systemic lupus erythematosus, or inflammatory lupus nephritis.

In an embodiment, administration of the compound comprises intralesional, intraperitoneal, intramuscular, or intravenous injection. In an embodiment, administration of the compound comprises infusion or liposome-mediated delivery. In an embodiment, administration of the compound comprises topical application to the skin, nasal cavity, oral membranes or ocular tissue.

The pharmaceutically acceptable carriers of the invention comprise any of the standard pharmaceutically accepted carriers known in the art. In an embodiment, the carrier comprises a diluent. In an embodiment, the carrier comprises a liposome, a microcapsule, a polymer encapsulated cell, or a virus. For example, in one embodiment, the pharmaceutical carrier may be a liquid and the pharmaceutical composition in the form of a solution. In another embodiment, the pharmaceutically acceptable carrier is a solid and the composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel or ointment and the composition is in the form of a suppository, cream, or liquid. Thus, the term pharmaceutically acceptable carrier encompasses, but is not limited to, any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as oil/water emulsions or triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules.

For example, tablets or capsules may utilize pharmaceutically acceptable binding agents (e.g. polyvinylpyrrolidone, hydroxypropyl methylcellulose, starch); fillers (e.g. lactose, microcrystalline cellulose, calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, silica or talc). Liquid preparations for oral administration may comprise syrups or suspensions prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. hydrogenated fats, sorbitol syrup), emulsifying agents (e.g. lecithin), and preservatives. Preparations may contain buffer, salts, and flavoring agents as appropriate. Suitable examples of liquid carriers include water, alcohols, and oils containing additives as described above.

When administered, compounds are often rapidly cleared from the circulation. Thus, in an embodiment, compounds are modified by the covalent attachment of water-soluble polymers such as polyethylene glycol (PEG), copolymers of PEG and polypropylene glycol, polyvinylpyrrolidone or polyproline, carboxymethyl cellulose, dextran, polyvinyl alcohol, and the like. Such modifications also may increase the compound's solubility in aqueous

solution, and reduce immunogenicity of the compound. Polymers such as PEG may be covalently attached to one or more reactive amino residues, sulphydryl residues or carboxyl residues. Numerous activated forms of PEG have been described, including active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxsuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-3 sulfone for reaction with amino groups, maleimido or haloacetyl derivatives for reaction with sulphydryl groups, and amino hydrazine or hydrazide derivatives for reaction with carbohydrate groups.

EXAMPLES

10 Features and advantages of the inventive concept covered by the present invention are further illustrated in the examples which follow.

Example 1: ELISA Assay Protocol

Generally, the protocol for detection of RAGE modulators is as follows. RAGE ligand (e.g. S-100b, β -amyloid, CML) is diluted to 5 μ g/ml in buffer A (fixing buffer) (100 mM Na₂CO₃/NaHCO₃, pH 9.8) and 100 μ l added to microtiter plate wells and allowed to incubate overnight at 4 °C to allow the ligand to become fixed to the surface of the wells. Wells are then washed 3 times with 400 μ l/well buffer C (wash buffer) (20 mM Imidazole, 150 mM NaCl, pH 7.2), with a 5 second soak in buffer C between each wash. Buffer B (blocking buffer) (50 mM Imidazole pH 7.2, 5% BSA, 5 mM CaCl₂, 5 mM MgCl₂) is then added to the wells and allowed to incubate for 2 hours at 37°C to block nonspecific protein binding sites. The blocking buffer is then aspirated from the wells, and the plate washed 3 times (400 μ l/well) with buffer C, with a 5 second soak in buffer C between each wash. The compound of interest, 25 μ l dissolved in 2% DMSO FAC (Final Assay Concentration) and sRAGE (75 μ l) (2.2×10^{-4} mg/ml FAC) are then added to each well, and incubated 1 hour at 37 °C. Meanwhile, polyclonal antibody or monoclonal antibody for sRAGE (e.g. 3.0×10^{-3} mg/ml and 1.9×10^{-4} mg/ml FAC, respectively), biotinylated goat F (ab')₂ anti-mouse IgG (e.g. 8.0×10^{-4} mg/ml FAC) (Biosource International, Camarillo, CA (TAGO)), and alkaline phosphatase labeled streptavidin (3.0×10^{-3} mg/ml FAC) (ZYMED, San Francisco, CA) are added to 5 ml of buffer D (complex buffer) (50 mM Imidazole, pH 7.2; 0.2% BSA, 5 mM CaCl₂, 5 mM MgCl₂) in a 15 ml conical tube and allowed to incubate 30 minutes at room temperature.

The solution containing sRAGE and the compound of interest is then aspirated from each well, and after 3 washes with wash buffer, with a 5 second soak between each wash, the anti-sRAGE:IgG:streptavidin-alkaline phosphatase complex is added to each well (100 μ l

complex per well). After 1 hour at room temperature, the solution in each well is aspirated, and the wells washed 3 times, with 5 second soaks between each wash.

The alkaline phosphatase substrate, *para*-nitrophenyl phosphate (pNPP) (1 mg/ml in 1 M diethanolamine, pH 9.8), is added and the color allowed to develop for 1 hr in the dark.

5 After the addition of 10 μ l stop solution per well (0.5 N NaOH; 50% methanol), the OD₄₀₅ is measured.

Example 2: Optimization of Assay Conditions

Experiments detailing the approach used for optimization of assay conditions are shown in Figures 3-6. For example, an experiment showing optimization of the assay for varying ligand concentrations is shown in Figure 3. Thus, using sRAGE at a concentration of 1.0×10^{-4} mg/ml FAC and polyclonal or monoclonal antibody (3.0 $\times 10^{-3}$ mg/ml and 1.9×10^{-4} mg/ml FAC respectively), it was found that maximal signal (1.06 OD) was achieved when S-100b at 500 ng/well was used for coating, and that ligand concentrations between 10 to 500 ng/well comprised a linear increase in sRAGE binding.

Experiments showing optimization of the assay at varying sRAGE concentrations are shown in Figure 4. Using 500 ng/well S-100b to coat the wells, anti-sRAGE polyclonal or monoclonal antibody (3.0 $\times 10^{-3}$ mg/ml and 1.9×10^{-4} mg/ml FAC respectively), and sRAGE ranging from 2 to 10 nM FAC, maximum signal is seen at about 10 nM sRAGE (Figure 4).

Experiments showing optimization of the assay for varying antibody concentration are shown in Figure 5. Wells were coated using S-100b at either 50, 500, 1,000 or 2,000 ng/well overnight as indicated. sRAGE at 6 nM was added and after 1 hour at 37°C, polyclonal antibody to sRAGE comprising final concentrations ranging from 5.0×10^{-2} to 5.5×10^{-5} mg/ml was added. It can be seen that regardless of the concentration of ligand used to coat the wells, maximal signal was obtained at 5.4×10^{-3} mg/ml antibody. Using 500 ng/well S-100b to coat the wells, and sRAGE at 1.0×10^{-4} mg/ml, maximal signal was found at 1.4×10^{-2} mg/ml antibody.

An experiment showing optimization of the assay for varying DMSO concentrations is shown in Figure 6. Thus, using sRAGE at a concentration of 6 nM FAC and polyclonal or monoclonal antibody (3.0 $\times 10^{-3}$ mg/ml and 1.9×10^{-4} mg/ml FAC, respectively), it was found that maximal signal was achieved using DMSO concentrations between 0% to 2% FAC.

Example 3: Accuracy and Precision of the Assay

In the experiment shown in Figure 10, wells were coated by incubation with 500 ng/well S-100b overnight, and after washing, sRAGE diluted in buffer D added to the final concentrations shown. Polyclonal anti-sRAGE antibody (3.0×10^{-3} mg/ml FAC) was then added, and after 1 hr at 37 °C, the plates washed and developed as described in Example 1. It
5 was found that over four separate determinations, the goodness of fit comprised an R^2 of 0.9383, with Kd value of 60 and 95% confidence interval of 41.2 to 78.6.

An assessment of signal variability is shown in Figure 7. In this experiment, 88 wells were coated by incubation with 500 ng/well S-100b overnight, and after washing, sRAGE diluted in buffer D added to all the wells with a final concentration of 2.2×10^{-4} mg/ml.
10 Polyclonal anti-sRAGE antibody (3.0×10^{-3} mg/ml FAC) was then added, and after 1 hr at 37 °C, the plates washed and developed as described in Example 1. An average OD₄₀₅ of 0.705 ± 0.054 and percent coefficient variance of 7.7 was observed. Data indicates that the signal variability from well to well is lower than 10%.

15 **Example 4: Detection of Low and High Affinity Binding of Ligands to sRAGE**

The utility of the assay for detecting compounds which antagonize both low and high affinity RAGE binding domains is shown in Figures 8-11. In these experiments, wells were coated with varying amounts of S-100b (50-500 ng/well overnight) and sRAGE added to each well. The amount of sRAGE bound to the immobilized ligand was detected using anti-
20 sRAGE polyclonal or monoclonal antibody (3.0×10^{-3} mg/ml and 1.9×10^{-4} mg/ml FAC, respectively) and the development system as described in Example 1. Plots were linearized by means of Scatchard analysis and values for Kd and Bmax determined. Statistical analysis of experimental precision was determined by utilizing GraphPad Prism version 3 software (GraphPad Software, Inc. San Diego, CA).

25 Both low and high affinity binding of sRAGE to S-100b are detected using the assay method of the invention. In the experiments shown in Figures 8 and 9, plates were incubated overnight with S-100b at a concentration of 50 and 100 ng/well, respectively. After washing, sRAGE comprising a final concentration ranging from about 0.3 to 10 nM was added to each well, and after 1 hour at 37 °C, bound sRAGE was measured by the addition of polyclonal antibody complex. For both experiments, a high affinity binding component comprising a Kd of about 6 nM (Figure 8) to 2 nM (Figure 9) is detected.
30

In the experiments shown in Figures 10 and 11, plates were incubated overnight with S-100b at a concentration of 500 and 100 ng/well, respectively. After washing, sRAGE

comprising a final concentration ranging from about 23 to 470, or 5 to 76 nM, respectively, was added to each well, and after 1 hour at 37°C, bound sRAGE measured by addition of the sRAGE polyclonal antibody complex (anti-sRAGE:IgG:streptavidin-alkaline phosphatase) and detection reagents. For both experiments, a low affinity binding site, having a Kd of
5 about 60 nM, was detected.

Example 5: Inhibition of sRAGE Binding by Modulators

Interaction of AGE's with RAGE generates intracellular oxidative stress (Yan, *et al.*, *J. Biol. Chem.*, 269:9889-9897 (1994)) resulting in the activation of the free radical-sensitive
10 transcription factor NF-κB and the activation of NF-κB regulated genes (Yan, *et al.*, *J. Biol. Chem.*, 269:9889-9897 (1994); Wautier, *et al.*, *Proc. Natl. Acad. Sci.*, 91:7742-7746 (1994)). Since RAGE transcription is also regulated by interaction at two different NF-κB binding
sites in the RAGE promoter sequence (Li, *et al.*, *J. Biol. Chem.*, 272:16498-16506 (1997)) an ascending detrimental spiral is fueled by this positive feedback loop. A secondary cell based
15 functional assay was used to evaluate small molecules which modulate binding of sRAGE to an immobilized ligand. The pNF-κB-Luc gene was introduced into a C6 glioma cell line expressing high levels of RAGE (*see e.g.* Huttunen *et al.*, *J Biol Chem.*, 274:19919-24 (1999)) by transfection using standard techniques. The pNF-κB-Luc reporter is designed such that upon activation of the NF-κB, the luciferase gene is expressed. Luciferase activity
20 is then quantified by measuring luminescence with a luminometer. Generally, expression of the luciferase gene is low. Upon introduction of a RAGE ligand, however, RAGE activates NF-κB, resulting in an increase in luciferase expression. Thus, the assay measures the ability of a compound to modulate RAGE induced-luciferase activity. If the modulator comprises an agonist, the compound induces activation of NF-κB transcription, and luciferase activity is increased. If the modulator is an antagonist, the compound inhibits the ability of RAGE
25 agonists to induce activation of NF-κB transcription, and luciferase activity is increased.

In the experiment shown in Figure 14, inhibition of S-100b/RAGE interaction in C6 glioma cells by Compound 1 (Figure 13) had an IC₅₀ of 3.3 μM. Thus, the cell based assay shown in Figure 14 showed good correlation with the binding ELISA IC₅₀ value for inhibition of sRAGE binding for Compound 1 of 1.8 μM (Figure 12).

With respect to the descriptions set forth above, optimum dimensional relationship of parts of the invention (to include variations in specific components and manner of use) are deemed readily apparent and obvious to those skilled in the art, and all equivalent relationships

to those illustrated in the drawings and described in the specification are intended to be encompassed herein. The foregoing is considered as illustrative only of the principal of the invention. Since numerous modifications and changes will readily occur to those skilled in the art, it is not intended to limit the invention to the exact embodiments shown and described, and
5 all suitable modifications and equivalents falling within the scope of the appended claims are deemed within the present inventive concept.

It is to be further understood that the phraseology and terminology employed herein are for the purpose of description and are not to be regarded as limiting. Those skilled in the art will appreciate that the conception on which this disclosure is based may readily be used as a basis
10 for designing the methods and systems for carrying out the several purposes of the present invention. The claims are regarded as including such equivalent constructions so long as they do not depart from the spirit and scope of the present invention.

What is claimed is:

1. A method for detection of RAGE modulators comprising:
 - 5 adsorbing a RAGE ligand onto a solid surface;
 - adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or a fragment thereof to the preadsorbed ligand;
 - adding anti-RAGE antibody which binds to RAGE protein or a fragment thereof;
 - determining the amount of RAGE protein or a fragment thereof bound to the ligand
- 10 by measuring the amount of anti-RAGE antibody bound to the solid surface; and comparing the amount of RAGE protein or a fragment thereof bound to the ligand in the presence of varying amounts of the compound of interest.
2. The method of claim 1, wherein the RAGE fragment comprises sRAGE as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto.
- 15 3. The method of claim 1, wherein the RAGE fragment comprises the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto.
4. The method of claim 1, wherein the RAGE fragment comprises a fragment of the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.
- 20 5. The method of claim 1, further comprising measuring the ability of the compound of interest to modulate the ability of RAGE comprising SEQ ID NO: 1 or a sequence substantially homologous thereto, to activate cellular processes.
6. The method of claim 5, wherein agonists comprise stimulation of RAGE-activated cellular processes and antagonists comprise inhibition of RAGE-activated cellular processes.
- 25 7. The method of claim 5, wherein the cellular process comprises activation of NF- κ B gene transcription.
8. The method of claim 1, wherein the antibody to RAGE protein, or a fragment thereof, comprises a monoclonal antibody.
- 30 9. The method of claim 1, wherein the antibody to RAGE protein, or a fragment thereof, comprises polyclonal antibody.
10. The method of claim 1, further comprising a second antibody which recognizes the anti-RAGE antibody.

11. The method of claim 10, wherein the anti-RAGE antibody and secondary antibody are allowed to complex prior to being added.
12. The method of claim 1, further comprising a colorimetric assay for the secondary antibody.
- 5 13. The method of claim 1, wherein the solid surface comprises a microtiter well.
14. The method of claim 1, wherein the solid surface comprises a dip-stick.
15. The method of claim 1, wherein the ligand comprises an advanced glycated endproduct, or fragment thereof.
16. The method of claim 1, wherein the ligand comprises carboxymethyl-lycine-modified AGE.
- 10 17. The method of claim 1, wherein the ligand comprises β -amyloid.
18. The method of claim 1, wherein the ligand comprises calgranulin.
19. The method of claim 1, wherein the ligand comprises S100b.
- 20 20. The method of claim 1, wherein the ligand comprises amphoterin.
- 15 21. The method of claim 1, wherein the compound of interest comprises a synthetic peptide.
22. The method of claim 1, wherein the compound of interest comprises a peptidomimetic.
23. The method of claim 1, wherein the compound of interest comprises an organic compound.
- 20 24. The method of claim 1, wherein the compound of interest comprises an inorganic compound.
- 25 25. The method of claim 1, wherein the compound of interest comprises a lipid.
26. The method of claim 1, wherein the compound of interest comprises a carbohydrate.
27. The method of claim 1, wherein the compound of interest comprises a nucleic acid.
28. Compounds for use as modulators of RAGE, wherein said compounds are identified by steps comprising:
 - adsorbing a RAGE ligand onto a solid surface;
 - adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or a fragment thereof to the preadsorbed ligand;
 - 30 adding anti-RAGE antibody which binds to RAGE protein or a fragment thereof;
 - determining the amount of RAGE protein or a fragment thereof bound to the ligand by measuring the amount of anti-RAGE antibody bound to the solid surface; and comparing

the amount of RAGE protein or a fragment thereof bound to the ligand in the presence of varying amounts of the compound of interest.

29. The compound of claim 28, wherein the RAGE fragment comprises sRAGE as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto.

5 30. The compound of claim 28, wherein the RAGE fragment comprises the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto.

10 31. The compound of claim 28, wherein the RAGE fragment comprises a fragment of the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.

32. The compounds of claim 28, wherein the method of identifying said compounds further comprises measuring the ability of the compound of interest to modulate the ability of RAGE, or a sequence substantially homologous thereto, to activate cellular processes.

15 33. The compounds of claim 32, wherein agonists comprise stimulation of RAGE-activated cellular processes and antagonists comprise inhibition of RAGE-activated cellular processes.

34. The compounds of claim 32, wherein a cellular process comprises activation of NF- κ B gene transcription.

20 35. The compound of claim 28, wherein the compound is used to treat symptoms of diabetes and symptoms of diabetic late complications.

36. The compound of claim 28, wherein the compound is used to treat amyloidosis.

37. The compound of claim 28, wherein the compound is used to treat Alzheimer's disease.

25 38. The compound of claim 28, wherein the compound is used to treat cancer.

39. The compound of claim 28, wherein the compound is used to treat inflammation.

40. The compound of claim 28, wherein the compound is used to treat kidney failure.

41. The compound of claim 28, wherein the compound is used to treat systemic lupus nephritis or inflammatory lupus nephritis.

30 42. The compound of claim 28, wherein the compound is used to treat erectile dysfunction.

43. A method for detection of RAGE modulators comprising:
adsorbing a RAGE ligand onto a solid surface;

adding a compound of interest and a protein comprising RAGE comprising SEQ ID NO: 1 or a fragment thereof to the preadsorbed ligand;

adding anti-RAGE antibody which binds to the RAGE or a fragment thereof, and a secondary antibody which binds to the anti-RAGE antibody;

- 5 measuring the secondary antibody bound to the anti-RAGE antibody; and
comparing the amount of RAGE bound to the ligand in the presence of varying amounts of the compound of interest.

44. The method of claim 43, wherein the RAGE fragment comprises sRAGE as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto.

10 45. The method of claim 43, wherein the RAGE fragment comprises the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto.

15 46. The method of claim 43, wherein the RAGE fragment comprises a fragment of the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.

47. The method of claim 43, further comprising measuring the ability of the compound of interest to modulate the ability of RAGE or a sequence substantially homologous thereto, to activate cellular processes.

20 48. The method of claim 47, wherein agonists comprise stimulation of RAGE-activated cellular processes and antagonists comprise inhibition of RAGE-activated cellular processes.

49. The method of claim 47, wherein the cellular process comprises activation of NF- κ B transcription.

50. The method of claim 43, further comprising a colorimetric assay for the secondary antibody.

25 51. The method of claim 43, wherein the RAGE antibody and secondary antibody are allowed to complex prior to being added to the reaction.

52. The method of claim 43, wherein the solid surface comprises a reaction vessel.

53. The method of claim 43, wherein the solid surface comprises a dip-stick.

54. A kit for detection of RAGE modulators comprising:

- 30 a solid surface comprising a RAGE ligand;
a separately packaged solution comprising a protein comprising RAGE comprising SEQ ID NO: 1 or a fragment thereof;
a separately packaged detection system comprising at least one agent which specifically binds to the RAGE or fragment thereof;

- separately packaged reagents for the detection system; and
instructions for use.
55. The kit of claim 54, wherein the detection system comprises a separately packaged antibody which binds to the RAGE, or fragment thereof.
- 5 56. The kit of claim 54, wherein the RAGE fragment comprises sRAGE as defined by the amino acid sequence SEQ ID NO: 2, or a sequence substantially homologous thereto.
57. The kit of claim 54, wherein the RAGE fragment comprises the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 3, or a sequence substantially homologous thereto.
- 10 58. The kit of claim 54, wherein the RAGE fragment comprises a fragment of the V-domain of RAGE as defined by the amino acid sequence SEQ ID NO: 4, or a sequence substantially homologous thereto.

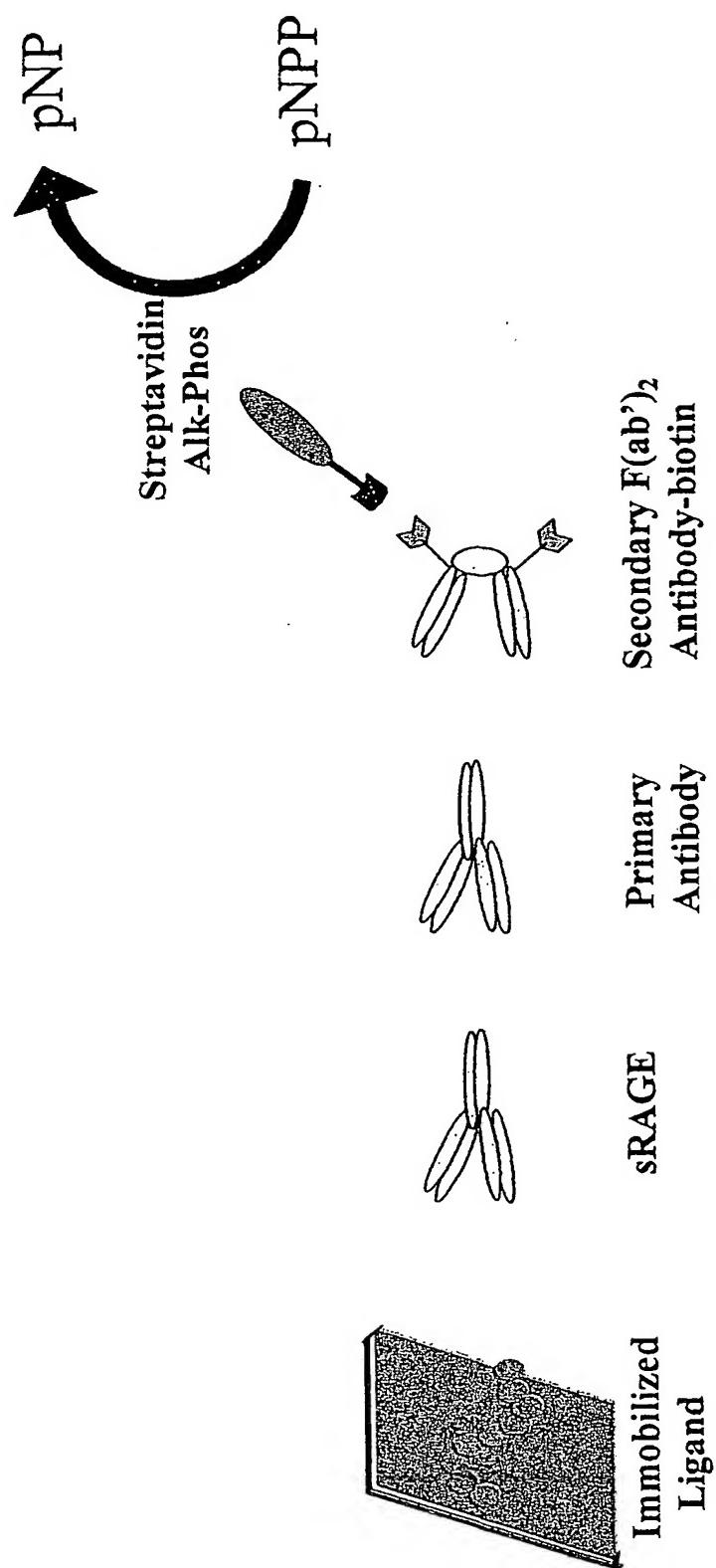


FIG. 1

A.

M91211: AMINO ACID SEQUENCE OF HUMAN RAGE (SEQ ID NO: 1)

GAAGTAVGAWVLVSLWGAVVGAQNIARIGEPLVLKCKGAPKK

PPQRLEWKLNTGRTEAWKVLSLWGAQNIARIGEPLVLKCKGAPKK

NRNGKETKSNYRVRVYQIPGKPEIVDSASELTAGVPNKVGTCVSEGSYPAGTLSWHL

GKPLVPNEKGVSVKEQTRRHETGLFTLQSELMVTPARGGDPRPTFSCSFSPGLPRHR

ALRTAPIQPRVWEVPLEEVQLVEPEGGAVAPGGTVTLTCEVPAQPSPQIHWMKDGV

PLPLPPSPVLLPEIGPQDQGTYSVATHSSHGPQESRAVSISIIEPGEEGPTAGS

GSGLGTIALALGILGGLGTAALLIGVILWQRRQRRGEERKAPENQEEEERAELNQSE

EPEAGESSTGGP

B.

AMINO ACID SEQUENCE OF HUMAN sRAGE (SEQ ID NO:2)

GAAGTAVGAWVLVSLWGAVVGAQNIARIGEPLVLKCKGAPKKPPQRLEWKLNTGRTEAWKV
LSPQGGGPWDSVARVLPNGSLFLPAVGIIQDEGIFRCQAMNRNGKETKSNYRVRVYQIPGKPEIV
DSASELTAGVPNKVGTCVSEGSYPAGTLSWHLGKPLVPNEKGVSVKEQTRRHETGLFTLQS
ELMVTPARGGDPRPTFSCSFSPGLPRHRALRTAPIQPRVWEVPLEEVQLVEPEGGAVAPGG
TVTLTCEVPAQPSPQIHWMKDGVPLPLPPSPVLLPEIGPQDQGTYSVATHSSHGPQESRAVS
SIIEPGEEGPTAGS VGGSGLG

FIG. 2

C.

AMINO ACID SEQUENCE OF THE V-DOMAIN OF HUMAN RAGE (SEQ ID NO: 3)

AQNITARIGEPLVLKCKGAPKKPPQRLEWKLNTGRTEAWKVLSHQGGPWDSVARVL

PNGSLFLPAVGIQDEGIFRCQAMNRNGKETKSNYRVRYQIPGKPEIVDSASELT

D.

AMINO ACID SEQUENCE FRAGMENT OF THE V-DOMAIN OF HUMAN RAGE (SEQ ID NO: 4)

AQNITARIGEPLVLKCKGAPKKPPQRLEWK

FIG. 2A

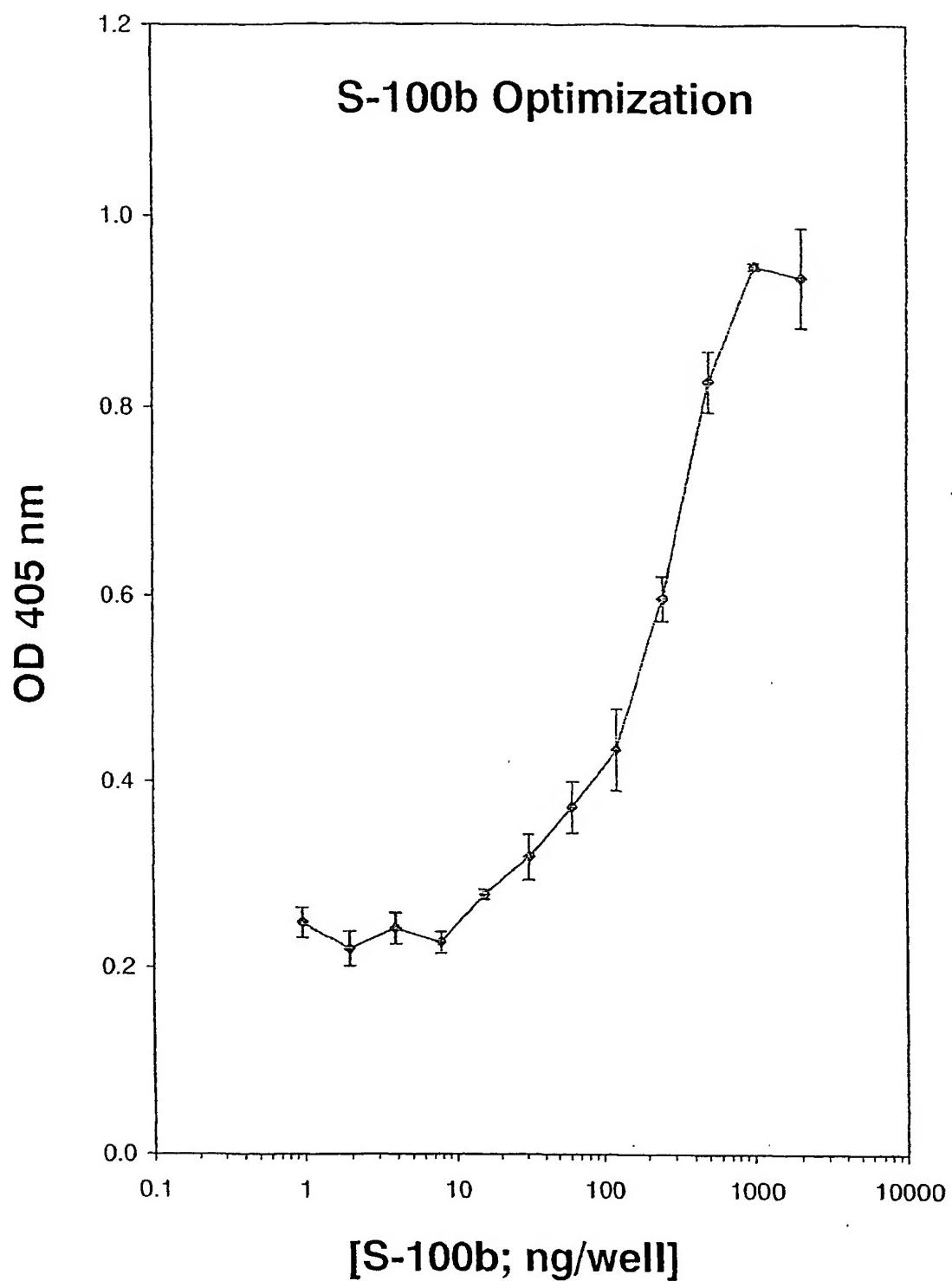


FIG. 3

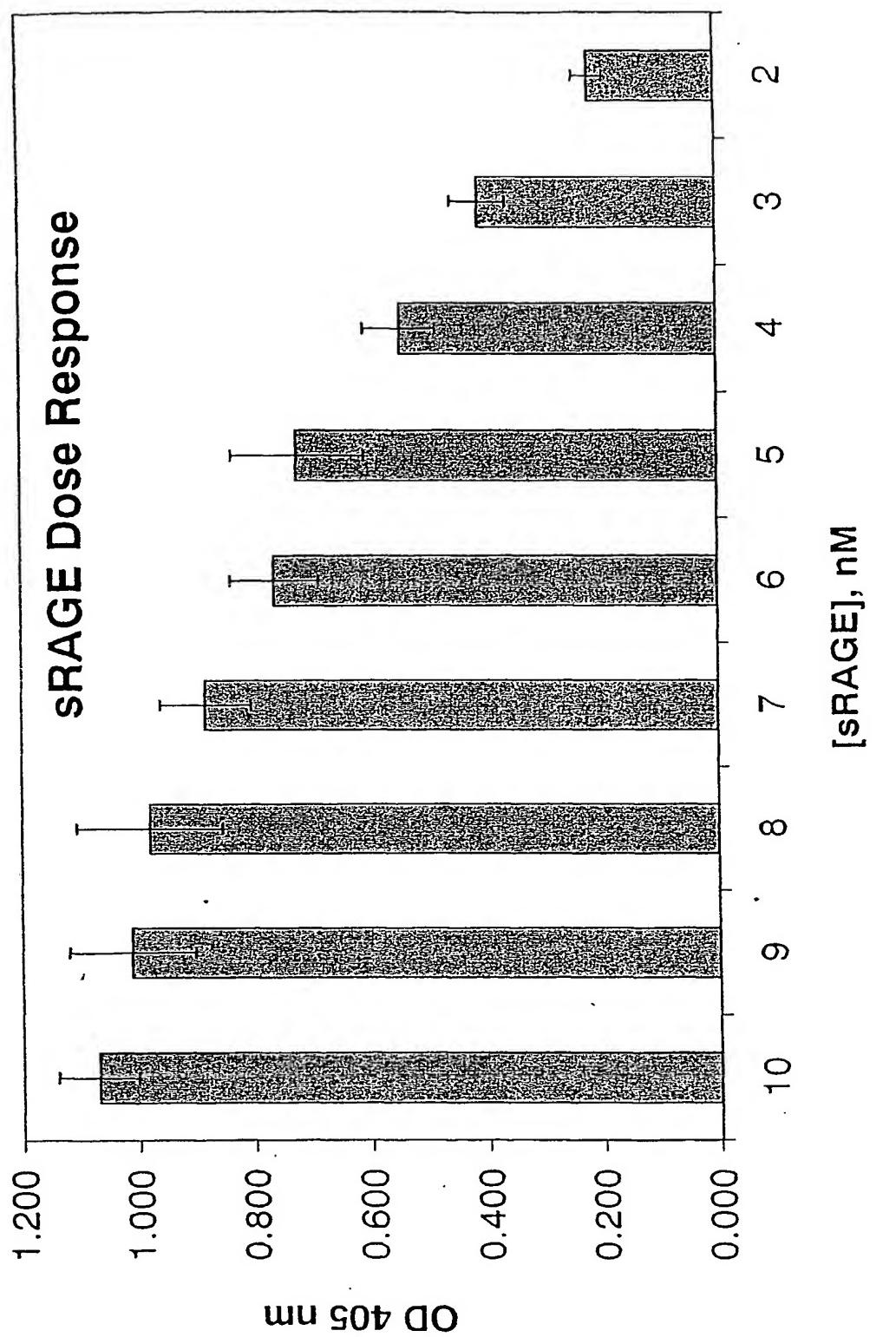
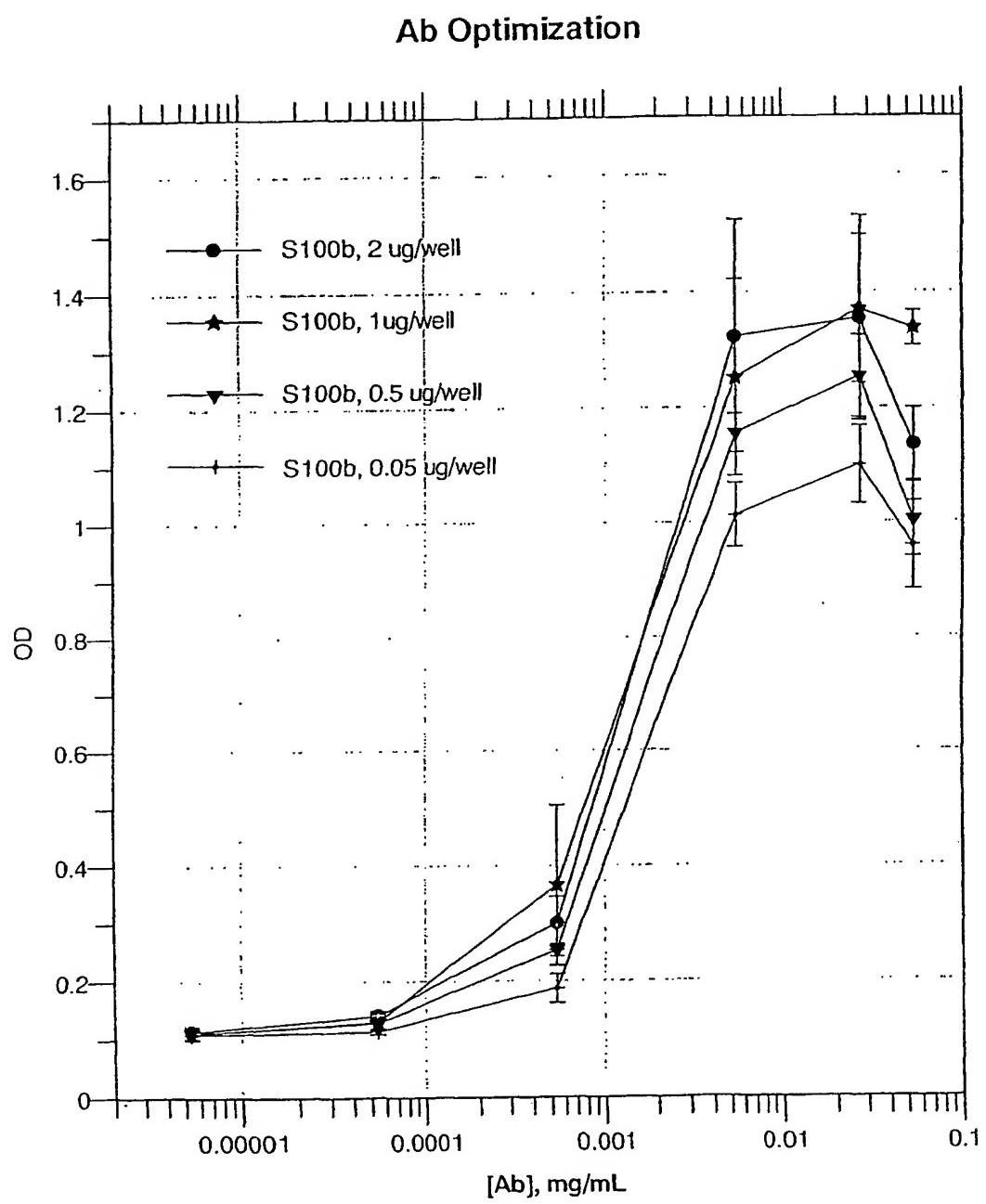


FIG. 4

**FIG. 5**

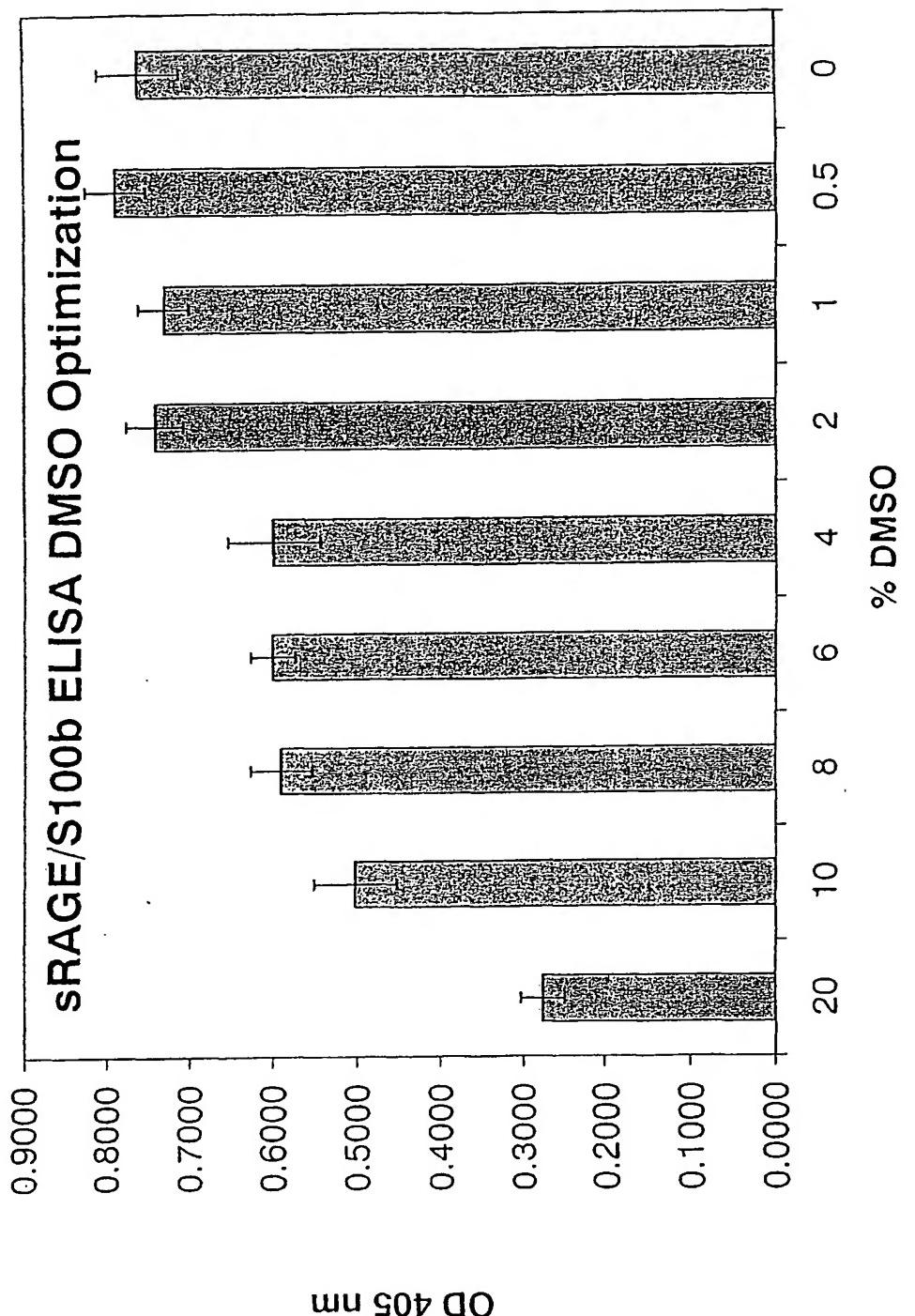


FIG. 6

sRAGE/S100b ELISA Signal Variability
AVE O.D = 0.705, SD = 0.054

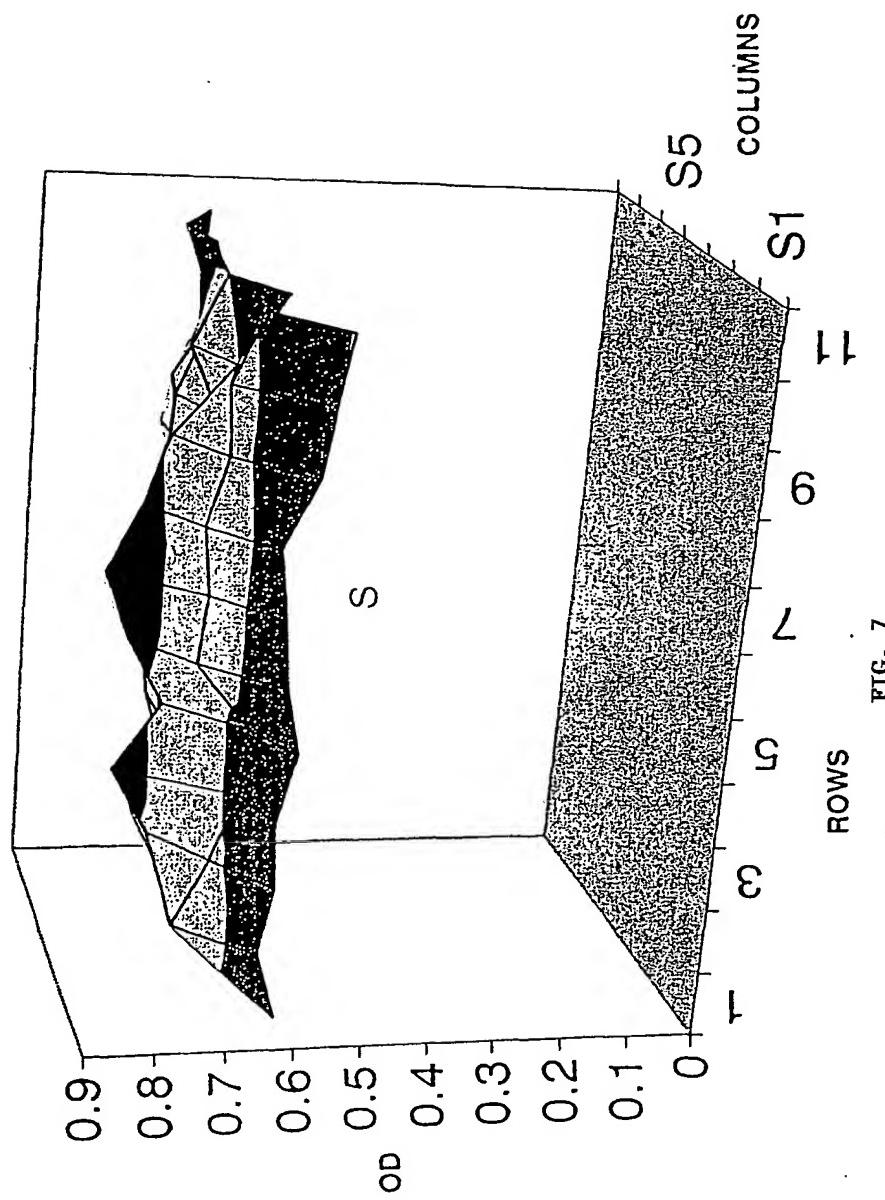


FIG. 7

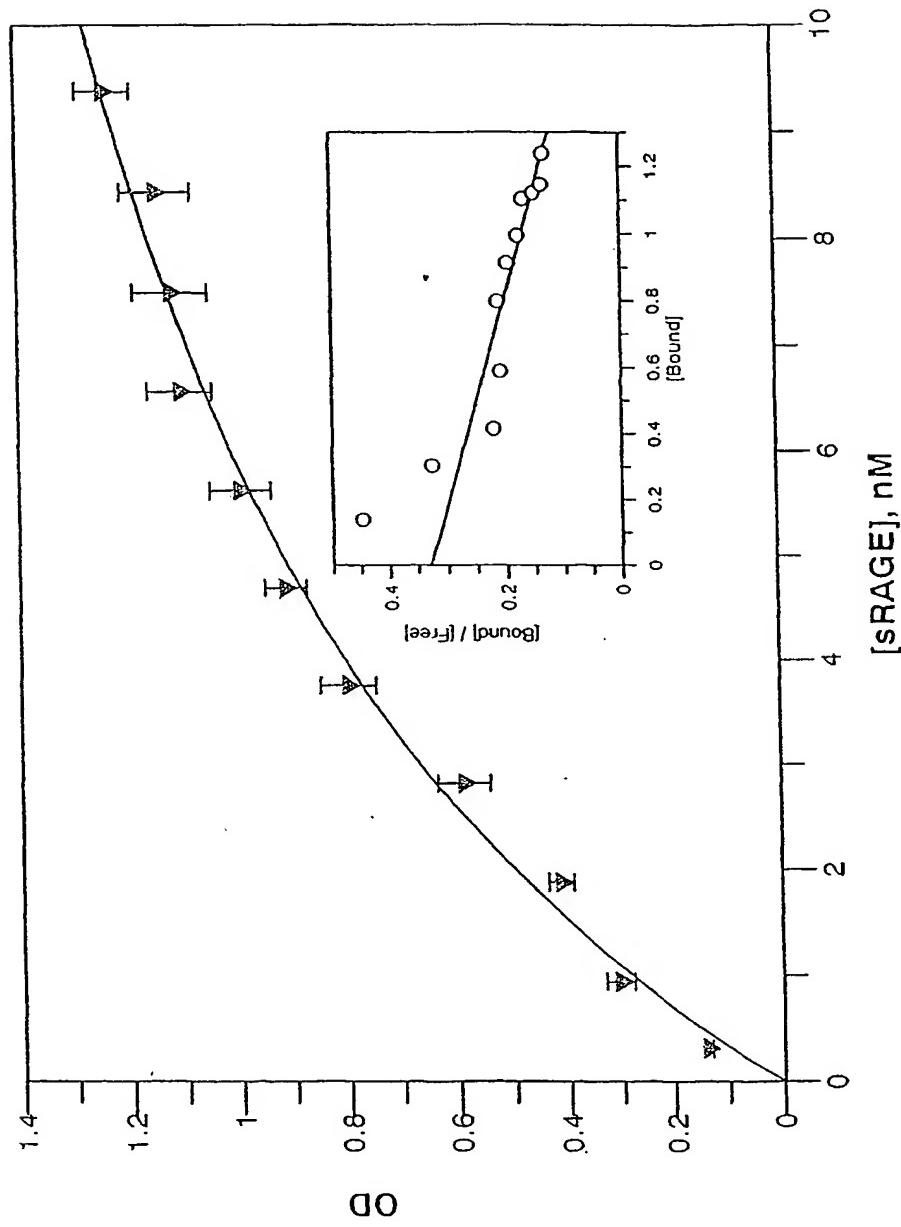


FIG. 8

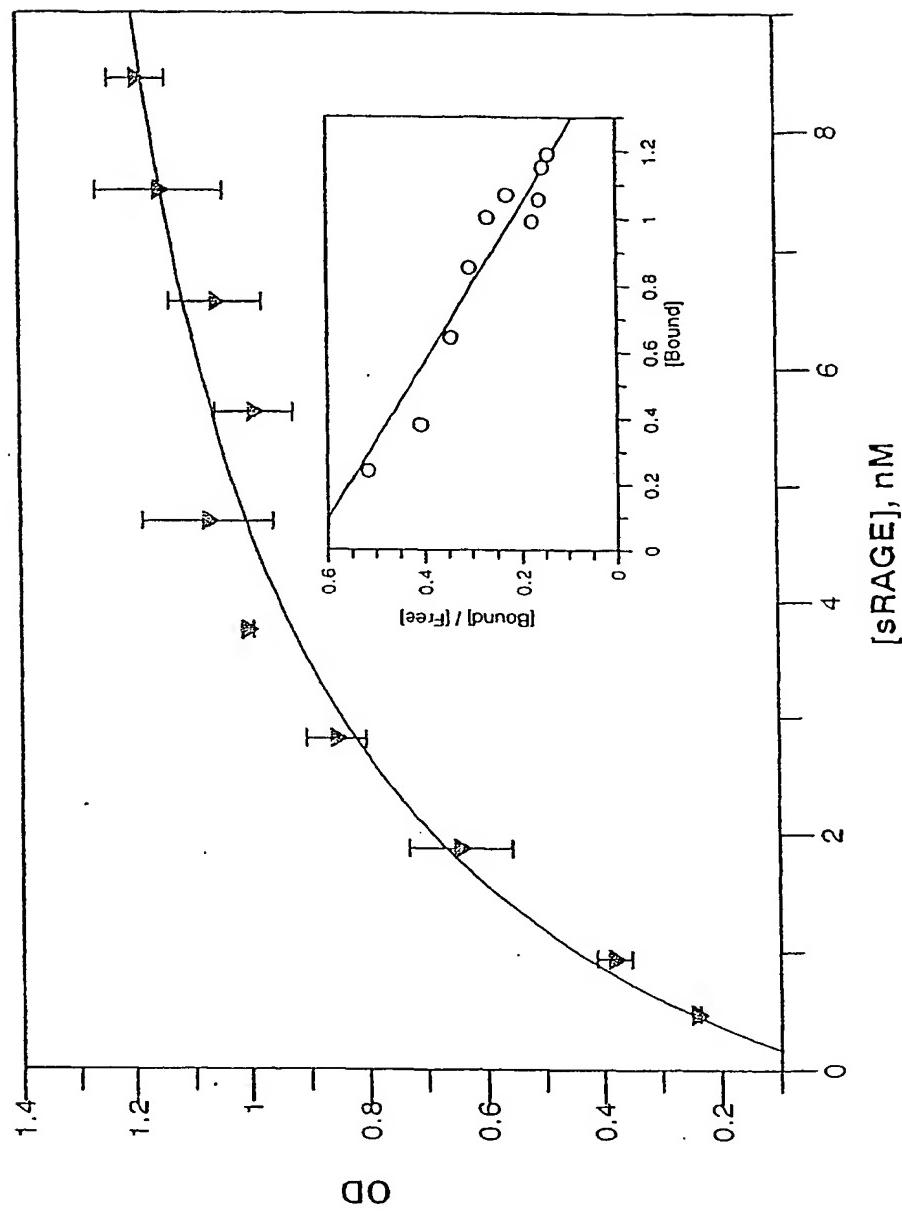


FIG. 9

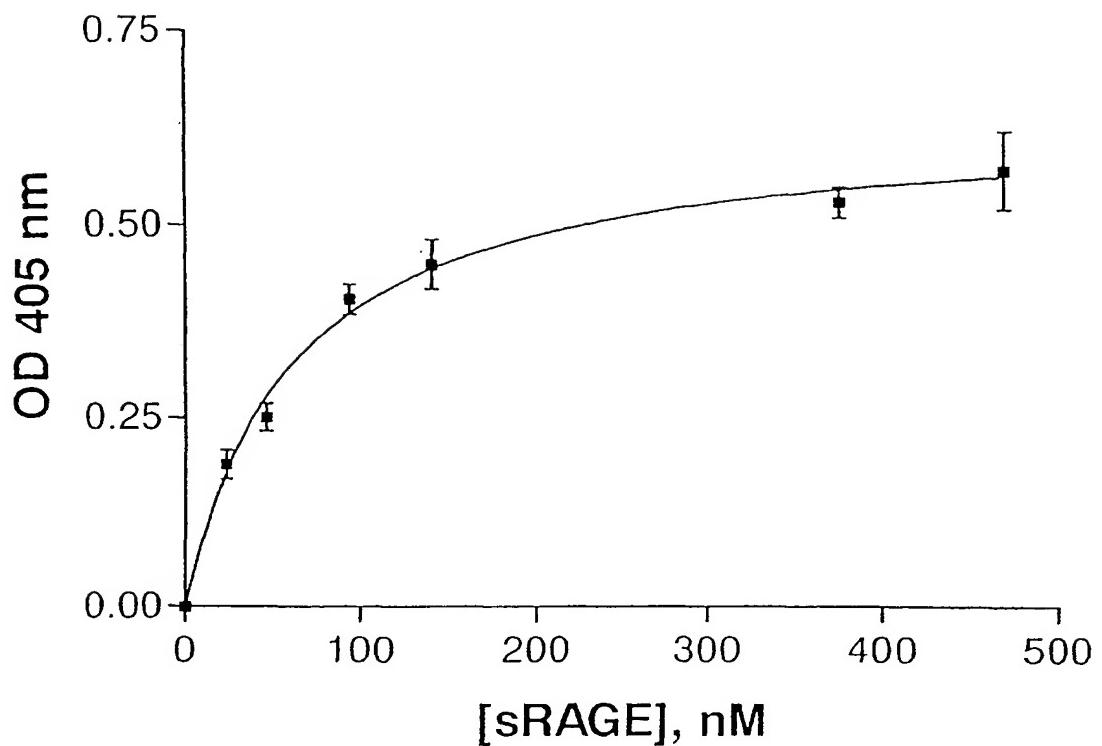


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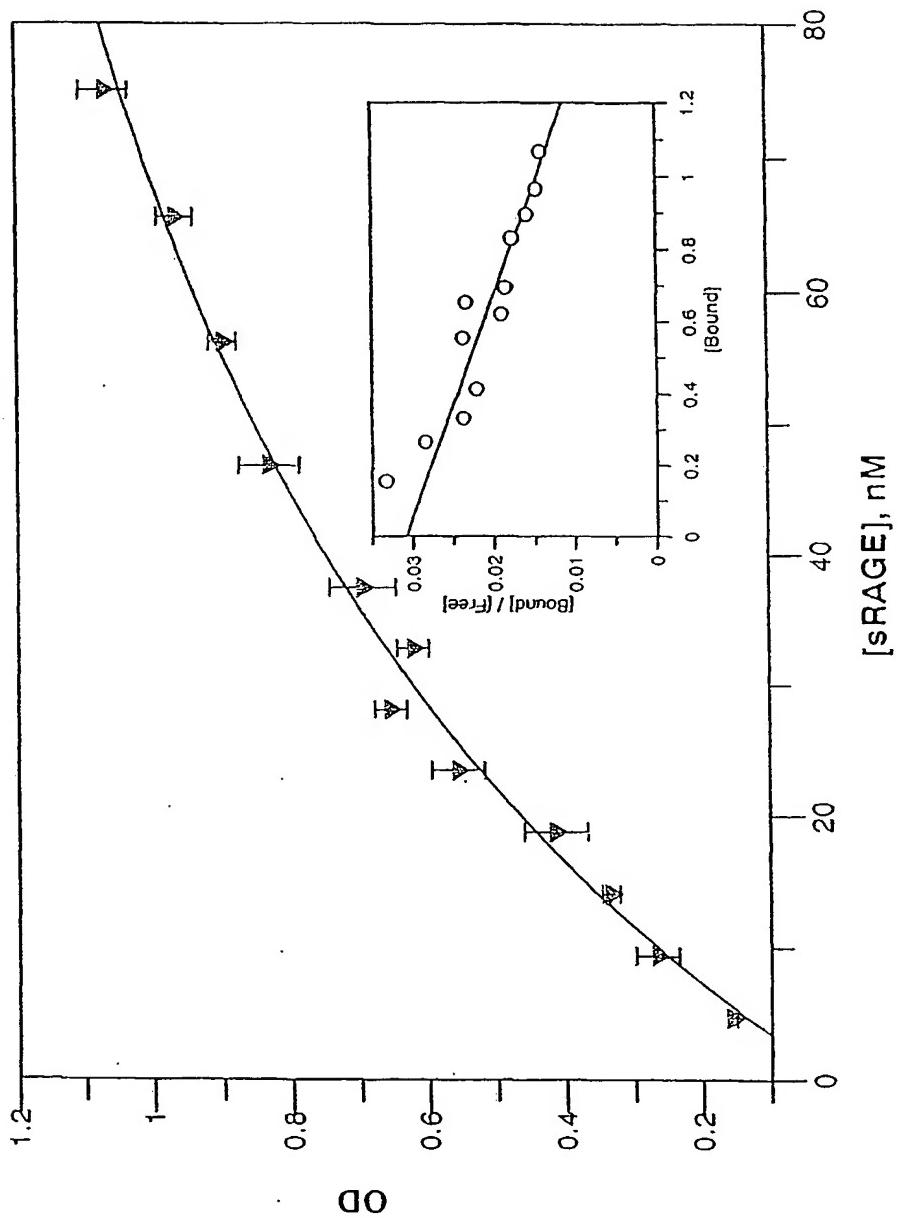


FIG. 11

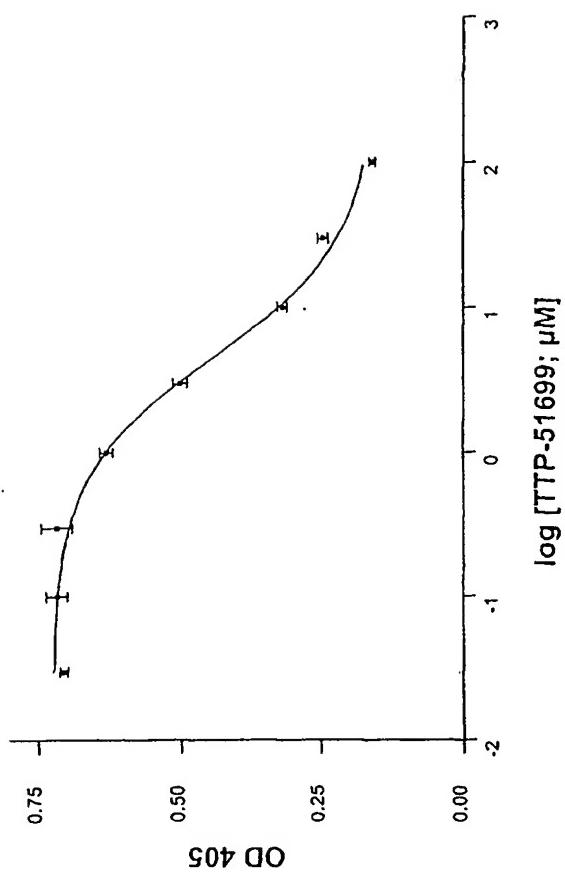


FIG. 12

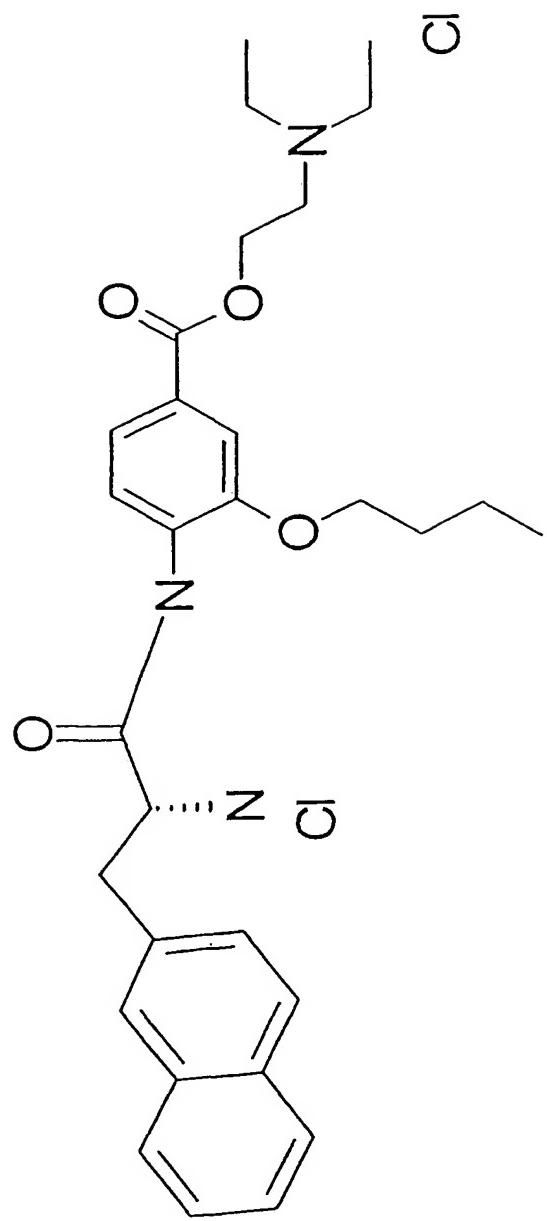


FIG. 13

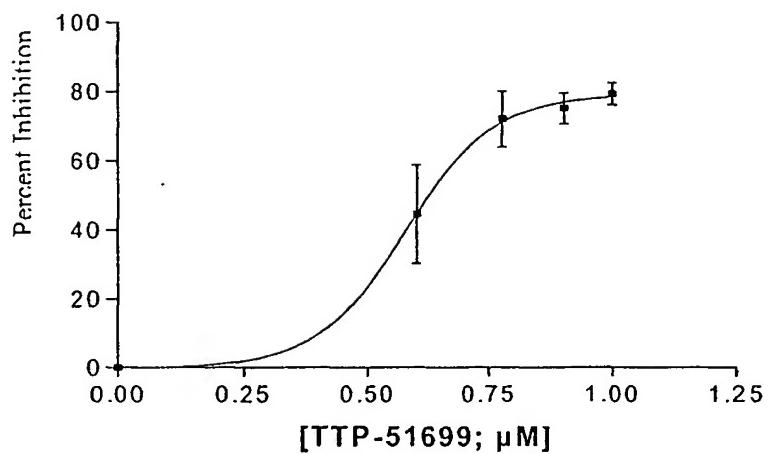


FIG. 14

1

SEQUENCE LISTING

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Leu Glu Trp Lys Leu Asn Thr Gly Arg Thr Glu Ala Trp Lys Val Leu
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Ser Pro Gln Gly Gly Pro Trp Asp Ser Val Ala Arg Val Leu Pro
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